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Amplified ribosomal DNA restriction analysis and repetitive element polymorphism-polymerase chain reaction (rep-PCR) DNA fingerprinting of members of *Frankia* genus

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Slow growing actinobacteria of the genus *Frankia* are best known for their nitrogen-fixing mutualism with dicotyledonous host plants called actinorhizal plants. Twenty nine (29) strains obtained from diverse host plants and geographic area, have been studied based on amplified ribosomal DNA restriction analysis (ARDRA) and repetitive element polymorphism-polymerase chain reaction (rep-PCR) DNA fingerprinting using BOX A1R primer. The collection has been classified into 28 ARDRA haplotypes clustered into three genogroups. The first genogroup 1 contains *Frankia* strains infecting *Elaeagnus*, genogroup 2 includes strains infecting *Casuarina*, while *Frankia* strains infective on *Alnus*, *Comptonia* and *Myrica* were grouped in genogroup 3. The results of BOX-PCR fingerprinting, supported the observation that BOX-PCR seems to be able to discriminate *Frankia* at strain level but are not useful for assigning strain to their respective genogroups or host infection groups.

Key words: *Frankia*, ARDRA, BOX-PCR, host infection groups.

INTRODUCTION

Frankia genus contains diazotrophic actinobacteria that are able to establish root nodules with diverse dicotyledonous host plants known as actinorhizal plants that have attracted interest with regard to the input of fixed nitrogen on marginal soil where indigenous legumes are absent (Gtari and Dawson, 2011). *Frankia* strains grow slowly with doubling times of 15 to 48 h or more leading to difficulty in arranging strains into phenotypically related groups (Benson and Silvester, 1993). However, the host-specific responses of the *Frankia* strains remain mostly the only useful criteria to group them into four major host infectivity groups (HSGs) (Backer, 1987). HSG 1 is composed of strains that infect *Alnus*, *Comptonia* and *Myrica*; HSG 2 strains infect members of

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the *Allocasuarina*, *Casuarina* and *Myrica*; HSG3 strains infect members of the Elaeagnaceae, Rhamnaceae, *Gymnostoma* and *Myrica* and HSG4 contained strains that nodulate members of the Elaeagnaceae but not *Myrica*. Phylogenetic analysis based on entire 16S rRNA gene sequences permitted to assign *Frankia* strains to four clusters (Normand et al., 1996). Cluster 1 includes *Frankia* strains which form nodules on members of Betulaceae, Myricaceae and Casuarinaceae. In cluster 2 are grouped *Frankia* strains that only infect members of the Coriariaceae, Datiscaceae, Rosaceae and Ceanothus of the Rhamnaceae. Cluster 3 strains form effective nodules on members of the Myricaceae, Rhamnaceae, Elaeagnaceae and Gymnostoma of the Casuarinaceae. Despite the fact that *Frankia* strains from cluster 1 and 3 are being routinely isolated and cultivated, those from cluster 2 have not been isolated in pure culture despite many attempts and remain, therefore, considered as an obligate symbionts. Atypical *Frankia* strains that are unable to infect or fix nitrogen are included in cluster 4. These clustering have been confirmed by other molecular studies such as intertranscribed spacers (ITS) 16S-23S rRNA (Ghodhbane-Gtari et al., 2010), *gyr*B (Nouioui et al., 2011) and *gln*II (Gtari et al., 2004; Nouioui et al., 2011) gene sequence analysis. Due to the slow growth of *Frankia* strains and the limited funding for maintaining several collections especially those containing unidentified and uncharacterized strains, there is need for reducing the costs of shortages without risk of losing biodiversity (Lumini and Bosco, 1999). As a result of this, the present study tests the efficiency of some genetic fingerprinting and low cost based techniques for worldwide and routine characterization of *Frankia* isolates.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Twenty nine (29) *Frankia* strains were used in the present study (Table 1). Cultures are routinely subcultured at 28°C in DPM medium (Baker and O'Keefe, 1984) modified to contain as carbon source in addition to Na-propionate, Na-pyruvate, Na-succinate, Na-acetate and glucose to accommodate strain specific requirements.

DNA extraction, PCR amplification and amplified ribosomal DNA restriction analysis

DNA extraction was made from one month old liquid culture of the *Frankia* strains after forcing several bacterial colonies (from a culture volume of 1-5 ml) through a 0.7 × 30 mm sterile needle to homogenize the mycelium. After centrifugation, the resulting cell pellet was washed twice with sterile distilled water, incubated for 30 min in DNA extracting buffer (100 mmol l^{-1} Tris-HCl, pH 8; 20 mmol l^{-1} EDTA, pH 8.2; 1.4 M NaCl, and 2% w/v cetyl trimethyl ammonium bromide (CTAB), chloroform extracted and ethanol precipitated. The DNA pellet was dissolved in 50 µl TE (10 mmol l^{-1} Tris-HCl, pH 8; 20 mmol l^{-1} EDTA, pH 8.2). PCR reaction of the 16S rRNA gene was carried out by using FGPS56-352 and FGPS1509'-153 following conditions described by Normand et al. (1996). PCR amplification were performed in 100 µl final reaction volume containing 10 ng genomic DNA, 1X Taq polymerase buffer, 1.5 mmol l^{-1} $MgCl_2$, 0.1 µM each dNTP, 0.2 µM each primers and 2 U Taq DNA polymerase. The thermal program consisted of three min at 95°C followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 45s. PCR products were digested with restriction enzymes; *Alu*I, *Hae*III and *Rsa*I, overnight at the optimal conditions recommended by the manufacturer. Repetitive element polymorphism-polymerase chain reaction (rep-PCR) was performed in 25 µl final volume using 50 ng genomic DNA, 1X Taq polymerase buffer, 2.5 mmol l^{-1} $MgCl_2$, 0.5 µM each dNTP, 0.5 µM BOX-A1R primer (Versalovic et al., 1994), 0.04 U μl^{-1} Taq DNA polymerase and 5% (v/v) DMSO, and subjected to a thermal program: 95°C for 5 min, 35 cycles consisting of 1 min at 94°C, 1 min at 45°C and 2 min at 72°C. Amplified ribosomal DNA restriction analysis (ARDRA) and BOX-PCR products were electrophoresed in 2.5% agarose in TBE buffer (Sambrook et al., 1989), ethidium bromide stained and photographed under ultraviolet light.

Fingerprints were analyzed using GelCompar II v. 6.5 (Applied Maths NV) and dendrograms were constructed using unweighted pair group method with arithmetic mean (UPGMA) algorithm based on Dice similarity coefficient for ARDRA and Pearson's correlation coefficient for BOX-PCR similarity matrix.

RESULTS AND DISCUSSION

Beside difficulties in isolating *Frankia*, the nitrogen-fixing actinobacteria and symbionts of actinorhizal plants, identifying and conserving diversity of cultured strains remain problematic in view of scarcity of financial supports in several still devoted laboratories in the field. Moreover, the non useful phenotyping methods especially the time consuming of plant infecting experiments suggest for developing a low cost and world widely used method for routine characterization of *Frankia* isolates. In the present study, 29 *Frankia* reference strains and isolates were characterized by ARDRA and repetitive element polymorphism-PCR (BOX-PCR) methods. Individual *Alu*I, *Hae*III and *Rsa*I restriction patterns of the 16S rRNA gene amplicon and UPGMA dendrogram were shown in Figure 1. The first genogroup 1 contains *Frankia* strains NRRL-B16219, NRRL-B16306, NRRL-B16316, NRRL-B16412, EAN1pec, BMG5.3, BMG5.11, BMG5.13 and BCU110501 infecting *Elaeagnus*, genogroup 2 includes strains; KB5 and CcI3 infecting *Casuarina*, while *Frankia* strains infective on *Alnus* (ArI3, AvcI1, AvsI1, ACN14a and ACN1), *Comptonia* (CpI1) and *Myrica* (NRRL-B16386) are grouped in genogroup 3. Moreover, this grouping based on ARDRA is driven by host plant infectivity rather than host plant origin of isolation. Some *Casuarina* (NRRL-B16306 and NRRL-B16412) and *Ceanothus* (NRRL-B16316) strains that are non infective on the latter host plant but infective on *Elaeagnus* (Baker, 1987) grouped accordingly to genogroup 1. Likewise strains isolated from different host plants that cross-infect the same plant sets are included in the same genogroup such as those isolated from *Alnus*, *Comptonia* and *Myrica* that cluster with *Alnus* infective strains. Such as CpI1 and NRRL-B16386 isolated

Table 1. *Frankia* reference strains and isolates used in this study.

Host infectivity	Registration	Acronym	Host plant of origin	Provenance	Reference
Alnus	ULQ010201401	ACN14a	*Alnus crispa*	Tadoussaq (Canada)	Normand and Lalonde, 1982
	ULQ0102001007	ACNl	*Alnus crispa*	Orléans (France)	
	HFP013003	Arl3	*Alnus rubra*	Oregon (USA)	Berry and Torrey, 1979
	DDB01020110	AvcI1	*Alnus viridis*	Ontario (Canada)	Baker et al., 1980
	LLR 160401	NRRL B-16386	*Myrica californica*	California (USA)	Lechevalier, umpublished
	DDB 01361310	NRRL B-16406 =AvsI4	*Alnus viridis*		Baker, 1987
	HFP070101	CpI1	*Comptonia peregrina*	Massachusetts (USA)	Callaham et al., 1978
	BMG5.40		*A. glutinosa*	Tunisia	This study
	BMG5.41		*A. glutinosa*	Tunisia	This study
	BMG5.42		*A. glutinosa*	Tunisia	This study
Elaeagnus	ULQ130100144	EAN1pec	*Elaeagnus angustifolia*	Ohio, U.S.A.	Lalonde et al., 1981
	DDB 000320	NRRL B-16219 = Cj	*Ceanothus jepsonii* (rhizosphere)	Wyoming, USA	Baker, 1987
	LLR 02022	NRRL B-16306 = R43	*Casuarina*		Baker, 1987
		NRRL B-16316= CaI1	*Ceanothus americanus*	Vermont, USA	Lechervalier umpublished
	LLR 020601	NRRL B-16412	*Casuarina equisetifolia*	Great Stirrup Cay, Bahamas	Lechervalier umpublished
	BCU110501	Dt501	*Discaria trinervis*	Argentina	Chaia, 1998
	BMG5.12	BMG5.12	*E. angustifolia*	Tunisia	Gtari et al., 2004
	BMG5.11	BMG5.11	*E. angustifolia*	Tunisia	Gtari et al., 2004
	BMG5.3	BMG5.3	*E. angustifolia*	Tunisia	Gtari et al., 2004
	BMG5.7	BMG5.7	*E. angustifolia*	Tunisia	This study
	BMG5.8	BMG5.8	*E. angustifolia*	Tunisia	This study
	BMG5.15	BMG5.15	*E. angustifolia*	Tunisia	This study
	BMG5.16	BMG5.16	*E. angustifolia*	Tunisia	This study
Casuarina	HFP020203	CcI3	*C. cunninghamiana*	Florida (USA)	Zhang et al., 1984
		KB5	*C. equesitifolia*	Kings bore (Australie)	Rosbrook et al., 1989
	BMG5.22	BMG5.22	*Casuarina glauca*	Tunisia	This study
	BMG5.20	BMG5.20	*Casuarina glauca*	Tunisia	This study
	BMG5.21	BMG5.21	*Casuarina glauca*	Tunisia	This study
	BMG5.23	BMG5.23	*Casuarina glauca*	Tunisia	This study
	BMG5.24	BMG5.24	*Casuarina glauca*	Tunisia	This study

from *Comptonia perigrena* and *Myrica californica* associated with *Alnus* strains or BCU110501 isolated from *Discaria trinervis* is grouped with *Elaeagnus* strains. The newly isolates from *Elaeagnus angustifolia* (BMG5.7, BMG5.8, BMG5.15 and BMG5.16), *Casuarina glauca* (BMG5.20, BMG5.22, BMG5.23 and BMG5.24) and *Alnus glutinosa* (BMG5.40, BMG5.41 and BMG5.42) grouped with their respective host plant groups. Repetitive element polymorphism-PCR using BOX- A1R primer applied for the 29 *Frankia* strains and isolates is shown in Figure 2. Twenty nine (29) unique profiles were obtained with one to three common bands. The remarkable differences observed by rep-PCR fingerprinting even among strains sharing the same ARDRA haplotypes

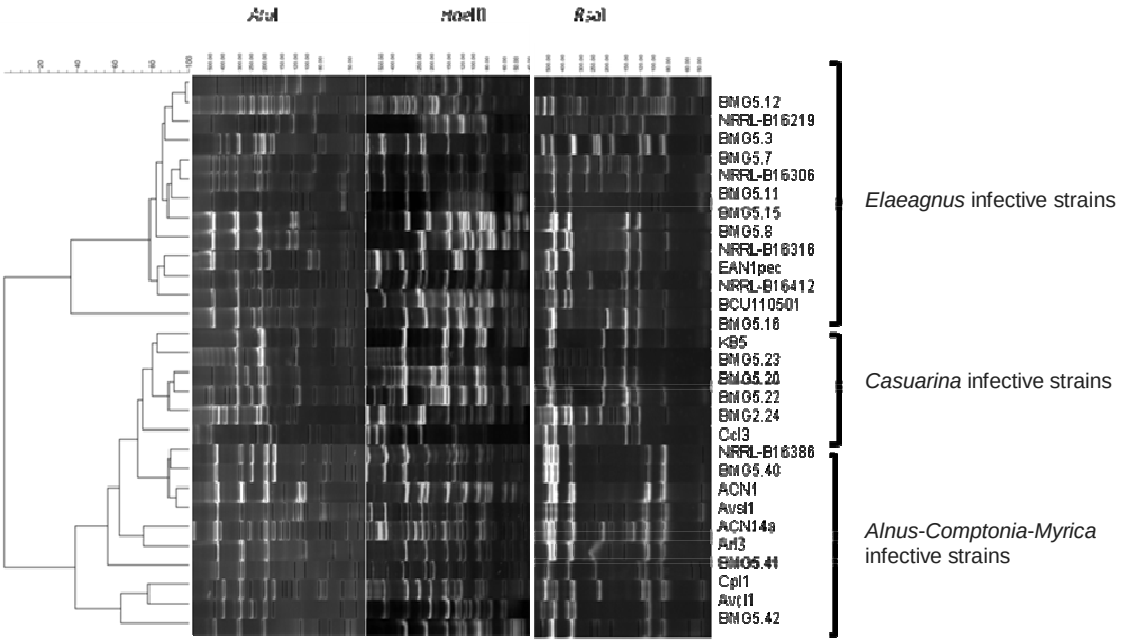

Figure 1. UPGMA cluster analysis using Dice coefficient of ARDRA digitized banding patterns generated by restriction digestions with *Alu*I, *Hae*III and *Rsa*I enzymes of the 16S rRNA gene amplicon from *Frankia* reference strains and isolates.

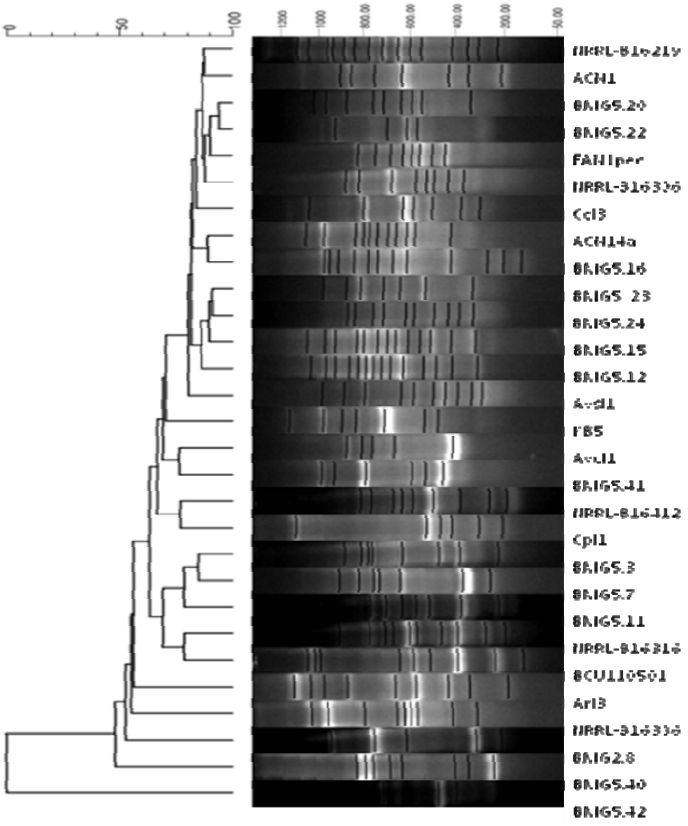

Figure 2. Cluster analysis of BOX-PCR fingerprints of *Frankia* reference strains and isolates using UPGMA algorithm based on Pearson's correlation coefficient.

(example *Casuarina* strains) indicate noteworthy genome variability among *Frankia* strains. While grouping is possible at high cutoff, the generated genogroups are not correlated to host infection groups that are determined in this study based on amplified ribosomal DNA restriction analysis. The high differences observed by BOX-PCR even on closely related *Frankia* strains was previously reported by Gtari et al. (2004), Murry et al. (1987) and Jeong and Myrold (1999) and seems to be a general feature among *Frankia* genus that reflect soil effect rather than host effect on *Frankia* genome variability. This suggests that repetitive element polymorphism-PCR using BOX-A1R primer is a reliable technique for strains discrimination among *Frankia* genus. Amplified ribosomal DNA restriction analysis has not been often used to characterize *Frankia* strains. Excepting Gtari et al. (2007) who performed an *in silico* ARDRA of entire diversity of *Frankia* 16S rDNA sequences retrieved from GenBank covering all host specificity groups and Huguet et al. (2004) who were interested only on Myricaceae isolated and uncultured strains directly in root nodules. Our ARDRA study may be the first report on assessment of *Frankia* diversity on a large collection of reference strains and isolates. The study demonstrated the feasibility and utility of ARDRA as fast and low cost based techniques for worldwide and routine characterization of *Frankia* isolates.

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